14-3-3 proteins interact with a 13-lipoxygenase, but not with a 9-lipoxygenase

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Abstract Associations between lipoxygenases (Lox) and 14-3-3 proteins were demonstrated by two different methods. First, immunoprecipitation experiments, using isoenzyme-specific monoclonal Lox antibodies, showed that 14-3-3 proteins co-precipitate with 13-Lox, but not with the 9-Lox from barley. Second, interactions between 13-Lox and 14-3-3 were established by surface plasmon resonance studies, showing that 13-Lox binds with 14-3-3 proteins in a concentration-dependent manner. The interactions between 14-3-3 proteins and 13-Lox may reveal their role during plant development.
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Key words: 14-3-3 protein; Lipoxygenase; Interaction

1. Introduction

Members of the highly conserved 14-3-3 protein family have been found to be associated with various proteins which play a role in cell cycle regulation, differentiation, coordination of signalling pathways and exocytosis [1,2]. Multiple isoforms of 14-3-3 proteins have been identified in several eukaryotic organisms [1–4]. For barley, three different isoforms of 14-3-3 have been reported, 14-3-3 A [5], 14-3-3 B (GenBank X93170) and 14-3-3 C (GenBank Y14200). There is little indication for functional specificity of individual isoforms of 14-3-3 proteins [6–8]. However, isoform-specific 14-3-3 antibodies showed different localisation of 14-3-3 isoforms in barley embryos during germination [9].

Several enzymes can be modified by 14-3-3 binding, amongst them nitrate reductase and sucrose phosphate synthase, i.e. key control enzymes of nitrate metabolism and carbon metabolism, respectively [3,4,7,10–14]. Furthermore, 14-3-3 target proteins have been identified as enzymes with potential roles in defence responses, including caffeate *O*-methyl transferase and ascorbate peroxidase [15,16]. A number of 14-3-3 binding motifs in target proteins have been described, all of which contain essential serine residues, such as RXY/FXSXP [17], RSXSXP [18], or RXSXSXP [19]. Interactions between 14-3-3 proteins and target proteins have been shown to be regulated often by reversible phosphorylation of serine or threonine residues within the binding motifs (summarised in [3,4]). Interactions between 14-3-3 proteins and the plant plasma membrane H⁺-ATPase, which maintains the electrochemical gradient across the plasma membrane, were shown to be stabilised by fusicoccin [8]. It has been suggested that 14-3-3 proteins function as a type of a molecular switch inducing a rapid change from one type of metabolism to another in response to a change in environment [4].

Lipoxygenases are a class of enzymes that are widely distributed in eukaryotes [20,21]. These enzymes play a key role in lipid metabolism and catalyse the first step in the dioxygenation of polyunsaturated fatty acids, containing a (1*Z*,4*Z*)-pentadiene structure, forming enantiomerically pure *S*-hydroperoxy fatty acids. In plants, lipoxygenases have been implicated in the biosynthesis of stress-responsive signalling molecules, such as traumatin [20] and jasmonic acid [22], and in the initiation of storage lipid degradation [23,24]. In barley, two lipoxygenase isoenzymes are present, Lox-1 and Lox-2. After incubation with linoleic acid, Lox-1 and Lox-2 form 9S- and 13S-hydroperoxide derivatives respectively, and therefore are termed 9-Lox and 13-Lox [25,26]. It has been suggested that each isoenzyme has different functions during germination. Lox-1 is present in quiescent as well as in germinating barley grains. Lox-1 is induced by jasmonic acid methyl ester or by wounding in green leaves of barley [27]. Lox-2 appears after germination and was suggested to be involved in the degradation of storage lipids during germination [23,24].

Since Lox is one of the key enzymes involved in lipid metabolism and stress signalling, we wondered whether lipoxygenases can interact with 14-3-3 proteins. If so, this would suggest that 14-3-3 binding is involved in the regulation of lipid metabolism and stress signalling. Comparison of barley Lox-1 and Lox-2 with common 14-3-3 binding motifs [17–19] points towards the sequence RKPSDSKP at position 234 of Lox-2 (GenBank accession number L37358) as putative 14-3-3 binding domain. In barley Lox-1 (GenBank L35931), the first serine within this domain is replaced by an asparagine, which may imply different physical behaviour. To investigate possible physical interactions between Lox isoenzymes and 14-3-3 proteins, both immunological and biophysical techniques were used, which show that 14-3-3 proteins interact with Lox-2, but not with Lox-1, from barley embryos. The possible implications of the interaction between 14-3-3 proteins and barley Lox-2 will be discussed.

2. Materials and methods

2.1. Plant materials and embryo imbibition conditions
Non-dormant grains (*Hordeum distichum* L. cv. Triumph) were obtained from Heineken Technical Services, The Netherlands (harvest

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Abbreviations: Lox, lipoxygenase

1989). Dormant grains (harvest 1997) were from genetically identical plants and were grown as described [28], with the modification that during ripening of the grains the plants were grown under a regime of 16 h at 12°C and 8 h at 10°C with 24 h light. For experiments, embryos were isolated from non-dormant grains which had been imbibed on two layers of Whatman No.1 paper in a Petri dish, containing 3 ml distilled water at 20°C in the dark. Alternatively, embryos were first isolated from dormant dry grains and then incubated in 300 μl distilled water, containing fusicoccin at a final concentration of 5×10^{-5} M, or ethanol at a final concentration of 0.05%.

2.2. Gel electrophoresis and Western blotting

SDS–PAGE was performed on 12.5% homogeneous gels. After electrophoresis, gels were electroblotted and the blots were cut into two parts. The upper section was incubated with Lox-1- or Lox-2-specific monoclonal antibodies (mAb, cell lines 5D2 and 4.2, respectively [29]), and the lower part was incubated with 14-3-3 A-, B- or C-specific polyclonal antibodies [9]. Immunoreactive proteins were detected after visualisation of anti-mouse or anti-rabbit conjugated alkaline phosphate with BCIP/NBT (Promega).

2.3. Heterologous expression of barley 14-3-3 protein in Escherichia coli

E. coli strain BL21(DE3) was transformed with the pET29b vector (Novagen), containing the coding sequences of the 14-3-3 cDNA clones (GenBank X62388, X93170 and Y14200 for 14-3-3 A, B and C, respectively). For surface plasmon resonance (SPR) experiments, proteins were purified after anion exchange chromatography, using a Resource Q column (Pharmacia). After equilibration of the column with 10 mM sodium phosphate buffer (pH 7.0), a linear gradient (0.01–1.00 M) of sodium chloride in 10 mM sodium phosphate buffer (pH 7.0) was used for elution. As a control, lysate was used from bacteria containing the pET29b vector without the cDNA insert. Peak fractions were dialysed against phosphate-buffered saline (PBS) and concentrated by freeze drying. 12.5% SDS–PAGE followed by silver staining and Western blotting was used to check for the quality of the preparations.

2.4. Purification of Lox-1 and Lox-2

For SPR experiments, Lox-1 and Lox-2 proteins were purified by affinity chromatography. 160 μg Lox-1- or Lox-2-specific mAb, cell lines 5D2 and 4.2 respectively, were coupled to 1 ml HiTrap NHS-activated columns (Pharmacia). Crude extracts from embryos, isolated from 4 day germinated non-dormant grains, were loaded onto the columns and after washing with 20 mM sodium phosphate buffer (pH 7.0), elution was performed with 0.1 M glycine–HCl (pH 2.7). Fractions were assayed for total Lox activity and peak fractions were dialysed against PBS and concentrated by freeze drying. 12.5% SDS–PAGE followed by silver staining and Western blotting was used to check for the quality of the preparations.

2.5. Immunoprecipitation

For immunoprecipitation experiments, Dynabeads M-280 (Dynal 112.02) were used, which were coated with sheep anti-mouse IgGs. 200 μl Dynabeads were loaded with a mixture of 80 μg Lox-1- and Lox-2-specific mAb (cell lines 5D2 and 4.2), and after extensive washing of the beads with PBST containing 0.1% NP40 and 1% bovine calf serum, the beads were incubated for 3 h at 4°C with extracts. Generally, extracts were prepared from 10 embryos, isolated from dormant barley grains which had been imbibed for 5 days, and subsequently were incubated with fusicoccin (5×10^{-5} M) for 16 h. Homogenisation of 10 embryos was carried out using 1 ml of 20 mM Tris–HCl buffer (pH 7.5), containing 0.1% NP40 and 0.5 mM phenylmethylsulfonyl fluoride. After washing, the beads were boiled directly in 200 μl sample buffer to release the immunoprecipitates, and 30 μl was applied to SDS–PAGE, followed by Western blotting analysis.

2.6. Surface plasmon resonance

SPR studies were performed using a Biacore X system (Biacore AB). 14-3-3 protein expressed in E. coli was covalently immobilised using amine coupling on a Biacore CM5 carboxymethyl dextran sensor chip, along with a similarly prepared bacterial control lysate. Approximately two-fold more protein from the 14-3-3 extract was linked to the chip in comparison with the control preparation, indicating the high level of recombinant 14-3-3 in the sample. Immunoaffinity-purified Lox-1 and Lox-2 preparations were passed over the immobilised proteins in concentrations ranging from 5 to 50 nM in running buffer (10 mM HEPES pH 7.4, 150 mM NaCl, 3 mM EDTA, 0.005% surfactant P20, 1 mg/ml carboxymethyl dextran), with or without the inclusion of 5 mM MgCl₂ or 10 μM fusicoccin as indicated, at a rate of 5 μl/min. Interactions were detected as changes in the SPR response, which is proportional to a change in mass at the sensor chip surface and therefore represents binding of analyte. Surfaces were regenerated using a 2 min pulse of 50 mM glycine pH 2.7 to remove bound protein before each analysis.
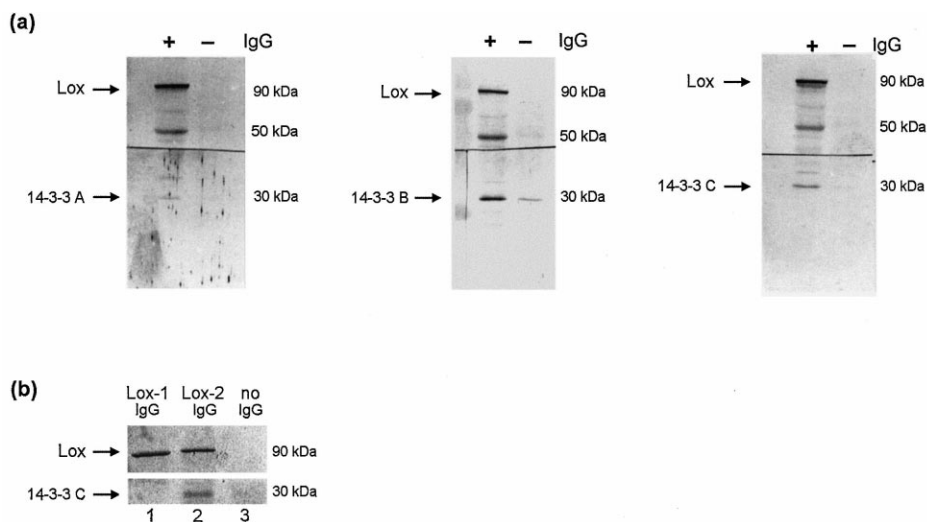

Fig. 1. a: Interaction of Lox and 14-3-3 proteins detected after immunoprecipitation. Immunoprecipitations using a mixture of Lox-1- and Lox-2-coated beads were performed as described in Section 2. As a control, extracts were incubated with unloaded beads (no Lox IgG). (Immuno)precipitates were analysed after SDS–PAGE and Western blotting. Upper panels of the blots were incubated with a mixture of Lox-1 and Lox-2 mAbs to detect Lox proteins, while lower panels were incubated with 14-3-3 A-, B-, or C-specific antibody to detect 14-3-3 proteins. Equivalent volumes were loaded in each lane. b: Lox-2, but not Lox-1, interacts with 14-3-3 proteins. Immunoprecipitation and subsequent analysis of the immunoprecipitates was performed as described previously with the following modifications. Beads were coated with either 80 μg Lox-1-specific mAb or 80 μg Lox-2-specific mAb (lane 1 and lane 2, respectively). As a control, extracts were incubated with unloaded beads (no Lox IgG; lane 3). Upper panels of the blot were incubated with Lox-1- or Lox-2-specific mAb (lanes 1 and 2 respectively), or a mixture of Lox-1- and Lox-2-specific mAbs (lane 3), while the lower panels of the blot were incubated with 14-3-3 C-specific antibody.

3. Results

3.1. Co-immunoprecipitation approach showing physical interactions between 14-3-3 proteins and barley Lox-2

It is generally accepted that co-immunoprecipitation of a protein is an indication that it forms associations with the protein that was immunoprecipitated primarily. Therefore, to investigate whether a direct physical interaction between Lox and 14-3-3 protein might exist, we performed immuno-precipitation experiments. Beads coated with a mixture of barley Lox-1 and Lox-2 mAbs were incubated with extracts isolated from dormant barley grains. Analysis of immune complexes was performed by SDS–PAGE and Western blotting. Our data show that the Lox mAbs exhibited the capacity to immunoprecipitate Lox proteins from crude extracts. Fig. 1A shows that Lox proteins exhibit molecular masses of about 90 kDa, while the band at about 50 kDa represents the antibody heavy chain. Further analysis of immune complexes revealed the antibody-dependent precipitation of 14-3-3 proteins (Fig. 1a, lower panel of the blots), indicating that 14-3-3 proteins form associations with lipoxygenases. Fig. 1a shows that 14-3-3 A, B, and C proteins all co-immunoprecipitate together with Lox protein. Although different levels of signal are obtained from the 14-3-3 A, B and C immunodetections, it is not possible to speculate on whether quantitative differences between Lox/14-3-3 interactions exist. For instance, antigenicity of 14-3-3 isoforms may be different, or the concentration or affinity of the anti-14-3-3 antibodies may not be the same. In controls, without Lox mAb coated to the beads, a weak signal of precipitated 14-3-3 proteins could also be observed (Fig. 1a). This background signal was significantly lower than the signal that was observed after precipitation using Lox mAb, and could largely be reduced by washing the beads with buffers containing 0.1% NP40.

Additional experiments were performed, using beads which were coated only with either Lox-1 or Lox-2 mAb. The mAbs used in the immunoprecipitation experiments, cell lines 5D2 and 4.2, had been shown previously to exhibit exclusive specificity towards Lox-1 and Lox-2 proteins, respectively [29]. Fig. 1b shows that both Lox-1 and Lox-2 proteins could be immunoprecipitated (lanes 1 and 2, respectively, upper panel of the blot). However, 14-3-3 proteins co-immunoprecipitated only using cell line 4.2, indicating that associations are formed between 14-3-3 and Lox-2 proteins only. For example, the interactions between Lox-2 and 14-3-3 C protein is shown in Fig. 1b, lane 2. In a reciprocal experiment, we performed immunoprecipitation experiments using tomato TFT4 14-3-3 antibodies (M.R. Roberts, unpublished) or mammalian 14-3-3 antibodies (Santa Cruz Biotechnology), under the same experimental conditions as described above. Analysis of the immune complexes revealed that only using TFT4 14-3-3 antibodies, barley 14-3-3 proteins could be immunoprecipitated (both 14-3-3 A, B, and C), although protein bands corresponding to 14-3-3 proteins on these blots were rather weak (data not shown). Furthermore, no co-immunoprecipitation of Lox-2 and 14-3-3 proteins could be observed. This may be due to structural hindrance, or just because insufficient amounts of 14-3-3 proteins were immunoprecipitated. The specific 14-3-3 A, B, and C anti-peptide antibodies, which were used in Western blotting experiments in the present paper, were not useful for our immunoprecipitation experiments, since they were shown to recognise 14-3-3 proteins under de-
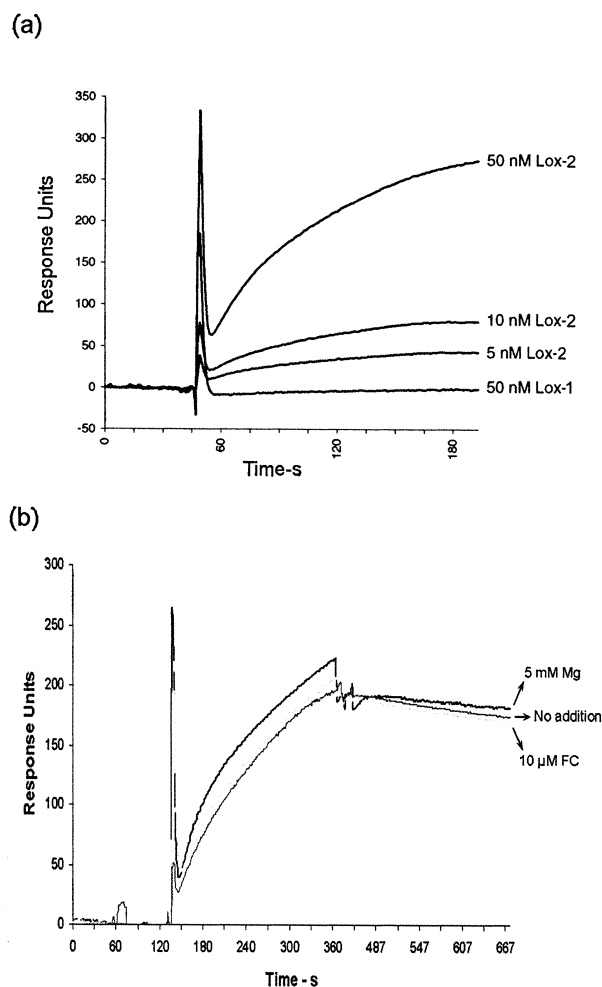

(a)

(b)

Fig. 2. a: Interactions between Lox and 14-3-3 proteins detected by SPR. Representative sensorgrams showing SPR responses from immobilised barley 14-3-3 C protein following the introduction of Lox-1 and Lox-2 proteins. Values plotted represent the difference between the separate measurements from the 14-3-3 C and control *E. coli* lysate sensor surfaces, over which the Lox proteins flowed sequentially. An increase in SPR response indicates binding of analyte to the 14-3-3 protein. The transient spikes in the responses around 50 s mark the times when the buffer flowing over the surfaces changed composition, representing the start of the injection of analyte. Only the association phase of the interaction is shown. b: Neither fusicoccin nor Mg^{2+} ions significantly affect associations between Lox-2 and 14-3-3 proteins. Representative sensorgrams showing SPR responses from immobilised barley 14-3-3 C protein following the introduction of 50 nM Lox-2 protein in the presence or absence of 10 μM fusicoccin or 5 mM $MgCl_2$. Although there is a small difference in the binding curve obtained for Mg^{2+}, analyses of several independent experiments suggest that neither association nor dissociation rates were significantly affected compared to the binding observed in the standard running buffer. The spikes just after 120 s represent the injection of analytes, after which association of 14-3-3 and analyte occurs until the drop in signal at around 360 s, which represents the end of the injection and a change back to running buffer, when dissociation begins.

naturing conditions only (C. Testerink and M.J. van Zeijl, unpublished data).

3.2. SPR showing physical interactions between 14-3-3 proteins and barley Lox-2

To verify and further investigate the interaction between 14-

3-3 and Lox proteins, real-time biomolecular interaction analysis using SPR was performed. Partially purified recombinant barley 14-3-3 protein expressed in *E. coli* was immobilised on a sensor surface, along with a similarly prepared untransformed bacterial lysate as a control. Immunoaffinity-purified barley grain Lox-1 and Lox-2 preparations were passed over the immobilised proteins and interactions were detected as changes in the SPR response. Fig. 2a shows that Lox-2, but not Lox-1, specifically binds to 14-3-3 C in a concentration-dependent manner. Similar data were obtained using recombinant barley 14-3-3 A and 14-3-3 B and tomato 14-3-3 TFT4 (data not shown). Neither fusicoccin nor magnesium ions (which stabilise some 14-3-3 interactions [13]) affected apparent rates of association or dissociation for the interaction between Lox-2 and 14-3-3 proteins detected using SPR (Fig. 2b).

4. Discussion

In the present paper, we show experimental data indicating a physical interaction between 14-3-3 proteins and a lipoxygenase from barley. Associations between Lox and 14-3-3 proteins were established by two methods, namely co-immunoprecipitation and SPR. Immunoprecipitations, using extracts of embryos isolated from dormant barley grains, indicate an association between Lox-2 (a 13-Lox), and 14-3-3 proteins (Fig. 1). A second method was used to verify the interaction, namely SPR, which can follow biomolecular interactions in real time. This method clearly confirms the interaction between 14-3-3 and Lox, and also shows that Lox-2, but not Lox-1, could bind to 14-3-3 proteins (Fig. 2a). The fact that 14-3-3 A, B and C proteins all co-immunoprecipitate and all interact with similar properties in SPR experiments is likely a result of the highly conserved character of the 14-3-3 protein family [17].

Neither immunoprecipitations nor SPR experiments give reason to believe that specificity of barley 14-3-3 isoforms exists with respect to their interactions with Lox-2. However, differences in spatial expression of 14-3-3 isoforms observed in germinating barley embryos [9] suggest that in planta functional isoform specificity may exist. Co-localisation studies using barley embryos may reveal isoform specificity of Lox-14-3-3 interactions in planta, and therefore will be part of our future research.

Because 14-3-3 binding motifs often contain essential serine residues, it is possible that the sequence RKPSDSKP at position 234 of Lox-2 is a putative 14-3-3 binding domain. In barley Lox-1, the first serine within this domain is replaced by an asparagine, which may explain its reduced affinity towards 14-3-3 proteins. Further investigations should confirm the predicted binding domain.

14-3-3 proteins have been shown to be in involved in the regulation of many cellular process in plant cells, regulating among others control enzymes of nitrate metabolism and carbon metabolism. Our data indicate that 14-3-3 proteins may also be involved in lipid metabolism. Barley Lox-1 (a 9-Lox) and Lox-2 (13-Lox) are the control enzymes of two different branches of the lipoxygenase pathway and the primary products, 9- and 13-hydroperoxide derivatives respectively, will lead to the formation of a different subset of lipoxygenase end-products. Our results suggest that only barley Lox-2, which is a 13-Lox, forms interactions with 14-3-3 proteins.

Our data raise the possibility that after binding to 14-3-3 proteins, 13-Lox forms protein–protein interactions, which may be consistent with the formation of oligomeric complexes of 15-Lox in animals after contact with membranes [30]. The 14-3-3-mediated action of 13-Lox in plants may affect not only enzymes which are involved in the initiation of germination, but also those in stress and pathogen responses. 13-Lox is involved in the biosynthesis of stress-responsive signalling molecules, such as jasmonic acid and traumatin, and 14-3-3 binding could provide a regulatory mechanism in the control of jasmonic acid- and traumatin-mediated events. Other enzymes with potential roles in defence responses have already been identified as 14-3-3 binding proteins [15,16]. It would be premature to speculate about the working mechanism of interactions between 13-Lox and 14-3-3 proteins. Our results should allow for future functional characterisation studies and further understanding in the regulatory mechanism of 14-3-3 proteins in lipid metabolism.

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